Regional Distribution of *Paenibacillus larvae* subspecies *larvae*, the Causative Organism of American Foulbrood, in Honey Bee Colonies of the Western United States

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ABSTRACT We examined honey bee, *Apis mellifera* L., colonies pollinating almonds in California during February 2003 for *Paenibacillus larvae* subsp. *Larvae*, the causative organism of the virulent brood disease American foulbrood. Colonies originating from the Rocky Mountain area and California had significantly higher numbers ($P < 0.05$) of bacterial colony-forming units (CFUs) (408 and 324 per 30 adult bees, respectively) than colonies from the upper Midwest (1.28). Colonies from the northwestern, central, and southwestern United States had intermediate CFU or bacterial colony levels. Operations positive for *P. larvae larvae* were relatively uniform at ≈70–80%, and no regional significant differences were found. Percentages of colonies with high CFUs (≥400 per 30 bees) differed significantly, with those from the Rocky Mountain region having 8.73% compared with those of the upper Midwest with 0%. The significance of CFU levels was evaluated by inoculating healthy colonies with diseased immatures and sampling adult bees. The number of CFUs detected per diseased immature was conservatively estimated to be ≈399 CFUs per 30 adult bees. We defined this spore level as 1 disease equivalent. Based on this, 3.86% colonies in our survey had 1 or more disease equivalent number of *P. larvae larvae* CFUs. Operations with high *P. larvae larvae* spore levels in their colonies will likely observe American foulbrood if prophylaxis is not practiced diligently.

KEY WORDS *Paenibacillus larvae*, foulbrood, migratory pollination

IN 2003, ≈1.4 MILLION COLONIES of honey bees were moved to the almond orchards of California (J. Traynor, personal communication). This is about one-half of the managed colonies in the United States. This massing of colonies allows surveys to be conducted economically. Comparatively high rental prices ($44–68 per colony) attract commercial beekeepers from across the United States, but ≈95% are from states west of the Mississippi River. During the past 3 yr, we have encountered operations with unexpectedly high levels of American foulbrood (AFB). We suspected that AFB levels rose along with the emergence of oxytetracycline (terramycin) resistance in the late 1990s and perhaps was aggravated by declining honey prices, which resulted in fewer colony inspections. Beekeepers knew they had a problem, but lacking an alternative treatment, continued to use oxytetracycline or attempted control with alternative techniques.

AFB is caused by the spore-forming, gram-positive bacterium *Paenibacillus larvae* subspecies *larvae.* It is a contagious disease of immatures that kills colonies if 100 or more cells are infected (Woodrow and States 1943). Annual losses in the United States are ≈$5,000,000 annually (Shimanuki 1990). Upon larval death, ≈2.5 billion spores are produced per bee (Sturtevant 1932). The sticky carcass is difficult for the workers to remove intact, and the bees eat it (Wilson 1972, Riessberger-Galle et al. 2001). Food transfers probably move spores of *P. larvae larvae* throughout the colony, including to susceptible immatures. *P. larvae larvae* spores are long lived with documented cases of 35 yr on record, albeit with reduced viability (Haseman 1961). Hygienic colonies rid themselves of the disease by rapidly uncapping and removing infected individuals (Rothenbuhler 1964, Spivak and Gilliam 1993). Eventually, many *P. larvae larvae* spores are eliminated from the colony either by defecation outside the colony or by normal adult attrition (Wilson 1971, Gochnauer 1981).

Examining colonies for AFB is laborious and disruptive to the colony. Even with a careful visual examination, diseased immatures in the early stages of infection are easily overlooked. Honey sampling provides a convenient diagnostic technique and has the advantage of pooling samples by apiary or by operation at the time of honey extraction (Hansen 1984, Von der Ohe and Dustmann 1997, Cox 2000). The disadvantage is that pooled samples do not provide colony

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incidence levels. They also lack positive identification of current disease status. Spores found in honey may originate from previous infections (Hornitzky and Clark 1991). Goodwin et al. (1996), Goodwin and Van Eaton (1999) described a technique using samples of adult bees collected from the broodnest and culturing the sample for *P. larvae larvae.* The relative noninvasiveness and ease of sampling allow large numbers of colonies to be examined. Our purpose was to characterize the *P. larvae larvae* spore levels in adult honey bees in commercial colonies of western United States and to determine their relationship with active AFB disease.

Materials and Methods

California Survey. Adult bees were collected from colonies pollinating almonds in California during February 2003. Colonies came from 16 states west of the Mississippi River and were located in the San Joaquin Valley between Bakersfield and Modesto, CA. We sampled 95 beekeepers. Maps of apiary locations were used to make selections. Within an apiary, six colonies were randomly selected. Approximately 100 bees were sampled from the broodnest, placed on dry ice, and shipped to Weslaco, TX. They were then held in a −20°C freezer until examined.

Bees were tested for *P. larvae larvae* by modifying the techniques of Goodwin and Van Eaton (1999) and Shimanuki and Knox (2000). Thirty bees were placed in a 55-ml test tube containing 15 ml of reverse osmosis water and macerated for ≈15 s with a Polytron tissue grinder (Brinkmann Intruments, Westbury, NY). Between samples, the grinder was cleaned and sterilized with two rinses each of Exspor (Alcide Corp., Redmond, WA), 70% ethyl alcohol, and reverse osmosis water. The bee-water slurry was heat shocked at 91°C for 20 min in a water bath. The slurry was cooled to ≈22°C. The clear portion was decanted and placed in a 5-ml sterile, capped culture tube, allowed to settle overnight, and decanted a second time. Samples were refrigerated (4°C) for short periods until tested. Culture medium (brain-heart infusion agar [BHIT], Difco, Detroit, MI) was prepared according to the Steinkraus and Morse (1996) and Shimanuki and Knox (2000). Culture plates were made by pouring ≈23 ml of BHIT into 100 by 15-mm petri dishes in a laminar flow hood. These were stored at 4°C until used.

The suspension of *P. larvae larvae* spores was agitated vigorously (Vortex, Scientific Industries, Bohemia, NY.) for 10 s. Then, 200 μl was pipetted onto a BHIT culture dish and dispersed with a sterile 25-mm cell spreader. Three culture dishes per sample were incubated at 34°C for 72–84 h in a darkened incubator. Colonies of *P. larvae larvae* were counted using a Leica Quebec darkfield colony counter (Buffalo, NY). When colony growth was too dense, serial dilutions of 10^{-1}, 10^{-2}, and 10^{-3} were made. The dilution that provided a dense, but discrete population (10–99 colonies) was counted.

Spore Recovery from Spiked Samples. Spore recovery from bees was evaluated by mixing ≈50,000 or 500,000 *P. larvae larvae* spores with 30 newly emerged, disease-free bees. Four treatments were used. 1) Negative control. Fifteen milliliters of reverse osmosis water was added to the bees and macerated for 15 s. Extraction, incubation, and counting were conducted as described above. 2) Positive control. Diluted *P. larvae larvae* stock inoculum (1 ml) containing ≈50,000 spores per milliliter (a) or 500,000 spores per milliliter (b) was heat shocked and then processed as described in treatment 1. 3) Adult bees processed according to the technique described by Goodwin and Van Eaton (1999). Water (15 ml) containing ≈50,000 (a) 500,000 (b) was added to a Ziploc-type bag containing the bees and crushed with a rolling pin for ≈20 s. The bag was suspended for 15 min and allowed to settle. The liquid portion was drained into a 5-ml test tube, heat shocked, and then processed as above. 4) Spores (≈50,000 or 500,000) were added to disease-free bees before grinding. One milliliter each of the two diluted *P. larvae larvae* stock inoculum described in two were added to 30 bees. The bees were then treated as those described in 1.

Spore Detection in Artificially Infected Colonies. The significance of spore counts in the survey was assessed by infecting healthy colonies ($n = 5$) with 0, 1, 10, 48.2, or 141 diseased immatures. In June 2003, 26 recently established colonies that were headed by newly mated queens (Wooten's Golden Queens, Palo Cedo, CA) were selected based on uniformity of adult population and broodnests. Colonies occupied two standard Langstroth hive bodies and had on average 10.9 ± 1.3 frames with adult bees. One month before testing, colonies were examined for *P. larvae larvae* and were found negative. Queens from these colonies were caged over a section of comb (14 by 11 cm) for 24 h and then released above a queen excluder. This comb with 400 eggs remained below the excluder. After 96 h, each newly hatched larvae was inoculated with ≈3,500 spores (Rinderer and Rothenbuhler 1969) by pipetting 2 μl of inoculum into the brood food surrounding the larva. In each of five colonies, either 0, 10, 20, 100, or 400 larvae were inoculated. Ten days after caging the queen, the capped brood was removed from the colonies and placed in an incubator (35 ± 2°C, 50% RH). Twenty days after oviposition, sealed brood was uncapped and diseased immatures were counted. The comb bearing the dead immatures was placed into their respective colonies. Treatments consisted of 0, 1, 10.0 ± 0.7, 48.2 ± 4.8, and 141.0 ± 41.9 diseased immatures. Approximately 100 adult bees were sampled at 3, 6, 12, 24, 48, 72, and 168 h after returning the dead immatures. For comparison, an additional colony received 50 cells of scale (dried remains of diseased immature). Testing for *P. larvae larvae* spores from 30 adult bees was done as described above. Eight days after infection, diseased combs were removed, and tylosin (200 mg of tylan, Elanco, Indiamapolis, IN, in 20 g of powdered sugar; Hitchcock et al. 1970; Peng et al. 1996; Alippi et al. 1999) was applied at weekly intervals for 3 wk. Because significant number of spores were still detected after 6 weeks, we applied an additional 2.5 g of tylan in 50 g of powdered

Table 1. Detection of *P. larvae larvae* bacterial CFUs in honey bee colonies originating from different geographical regions in the United States

Region/state	No. colonies	No. colonies *P. larvae larvae* positive	% positive	*P. larvae larvae* CFUs, mean ± SD
Rocky Mountain[a]	126	49	38.9	408.1 ± 2614.8a[b]
California	234	91	38.9	324.1 ± 3639.5a
Southwest[c]	30	9	30.0	139.0 ± 676.4ab
Northwest[d]	54	11	20.4	73.0 ± 366.5ab
Central[e]	30	9	30.0	29.5 ± 105.9ab
Upper Midwest[f]	96	21	21.9	1.28 ± 9.5b

Detection was by sampling 30 adult bees from the broodnest and culturing the bacterial flora for *P. larvae larvae.* To estimate the number of bacterial spores, data should be multiplied by about a factor of 20.

[a] Colorado, Montana, Utah, and Wyoming.

[b] Means followed by the same letter are not significantly different ($P > 0.05$) according to LSD.

[c] Arizona and Texas.

[d] Washington, Oregon, and Idaho.

[e] Missouri, Nebraska, and Kansas.

[f] North Dakota, South Dakota, and Minnesota.

sugar, and a 227 g of grease patty (6:4:1, powdered sugar/Crisco/sterilized honey) containing 1.0 g of tylan. Bee samples were taken from the broodnest at 1, 2, 6, and 12 wk of the initial tylosin treatment.

Analysis of variance (ANOVA) was performed on log-transformed spore levels found in colonies from different geographical regions and evaluated with least significant difference (LSD). χ^2 analysis was used to evaluate differences in the regional distribution of colonies admitted by the California Department of Food and Agriculture (CDFA) entry checkpoints and those sampled in our survey. Differences in CFU counts among treatments in the artificial colony infection experiment were examined with ANOVA and evaluated with LSD. Correlation coefficients were calculated for CFUs detected, and distance sample was taken from the infected honeycomb.

Results

California Survey. Of the 570 colonies sampled, 33.3% were found positive for *P. larvae larvae* spores (Table 1). The highest percentage of positive colonies came from the Rocky Mountain states and California (both 38.9%). The lowest (20–22%) was found in the upper Midwest and Pacific Northwest. The trend in colony spore levels paralleled the prevalence of infected colonies. Colonies from the Rocky Mountain states had on average 408.1 CFUs and those from California had 324.1, which was significantly higher ($P < 0.05$; LSD) than colonies from the upper Midwest (mean = 1.28). Colonies from the Pacific Northwest, central, and southwestern states had an intermediate number of CFUs (29.52–138.96). Colonies positive for *P. larvae larvae* had levels ranging from 1 to 53,000 CFUs. Their distribution is shown in Fig. 1.

We surveyed 95 beekeepers. This is ≈10% of the of beekeepers who pollinated almonds in 2003, although an exact count is difficult to determine. Approximately 50% of the colonies were from California (J. Traynor, Scientific Ag, and E. Mussen, University of California, personal communications). Table 2 shows that 41.1% of surveyed colonies originated from California, 22.1% from the Rocky Mountain region, and 16.8% from the upper midwestern states. The CDFA check stations authorized entry for 754,697 colonies, which indicates that ≈645,303 colonies came from within California. The distribution of surveyed colonies was significantly different from the CDFA data (Table 2; $\chi^2 = 373.4, n = 6, P < 0.05$). Our survey overrepresented the Rocky Mountain region (+12.8%) and underrepresented the Pacific Northwest (−8.0%). The percentage of beekeepers from the different geographical regions that had at least one colony positive for *P. larvae larvae* was relatively uniform and ranged from 68.7 to 80%.

Spore Detection in Spiked Samples. We found that *P. larvae larvae* spores exposed to macerated bees (treatment 4) produced significantly more CFUs ($P <$

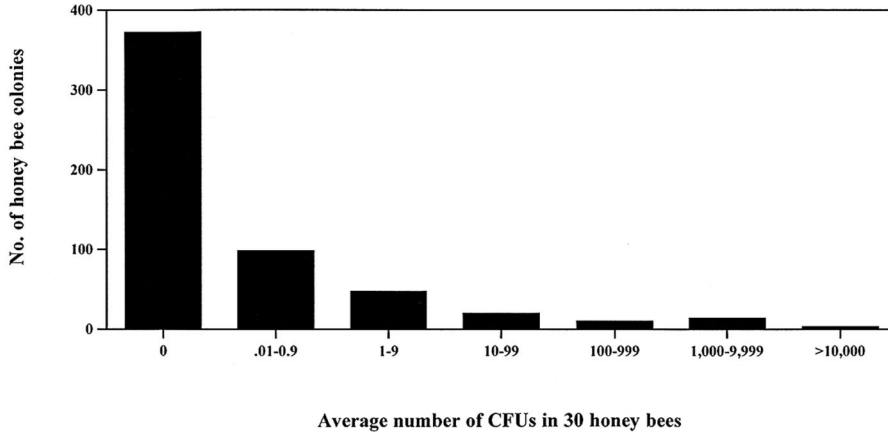

Fig. 1. Abundance of *P. larvae larvae* spores in 30 adult honey bees sampled from colonies pollinating almonds in California, 2003.

Table 2. Percentage of colonies represented in survey and percentage of beekeepers positive *for P. larvae larvae*

Region/state	% colonies CDFA checkpoint data[a]	% colonies in USDA survey[b]	No. beekeepers tested	% positive for *P. larvae larvae* spores
Rocky Mountain[c]	9.3	22.1	21	71.4
California	46.1	41.1	39	71.8
Southwest[d]	2.1	5.3	5	80.0
Northwest[e]	17.5	9.5	9	77.8
Central[f]	0.5	5.3	5	80.0
Upper Midwest[g]	19.2[h]	16.8	16	68.7

[a] Based on ≈1,400,000 colonies.
[b] Based on 570 colonies (areas with asterisk, below, not represented in USDA survey). Colony distribution in USDA survey significantly different than CDFA colony count ($P > 0.05$) as determined by χ^2 analysis. This may have been caused by not sampling in northern California. Additionally, some colonies may have crossed into California unchecked.
[c] Colorado, Montana, Utah, and Wyoming.
[d] Arizona, Texas, and New Mexico.
[e] Washington, Oregon, and Idaho.
[f] Missouri, Nebraska, Kansas, and Iowa.
[g] North Dakota, South Dakota, Minnesota, and Wisconsin.
[h] An additional 71,157 colonies came from New England and 294 from Michigan. The regional distribution of colonies in survey and CDFA data was significantly different ($\chi^2 = 373.4$, $n = 6$, $P < 0.05$).

0.05; LSD) than the technique described by Goodwin et al. (1996; treatment 3). On average, 4,162 (8.3%) and 14,778 (3.0%) CFUs were observed in treatment 4a and 4b, respectively, compared with 508 (1.0%) and 5,133 (1.0%) of treatment 3a and 3b, respectively. This represents about a three- to eight-fold increase in CFUs (to convert CFUs to spore numbers, they should be multiplied by ≈20). When *P. larvae larvae* spores were inoculated directly onto BHIT media without contact with the bees (positive control, treatment 2a and 2b), detection dropped to 94 (0.2%) and 2,320 (0.1%) CFUs for the 50,000 and 500,000 stock suspension, respectively.

Spore Detection in Artificially Infected Colonies. Colonies removed the caps from all diseased cells within 3 h of placing the AFB comb in the colony. This was associated with relatively high levels of spores in adult bees, especially those from colonies receiving the highest number of diseased immatures (Fig. 2). Spore numbers were low in all treatment groups at the 6-h period. Dead removal was first noted at the 6-h observation. Colonies that received 1, 10, 48.2, and 141 diseased immatures required ≈9.6, 31.2, 67.2, and 76.8 h, respectively, to remove all the dead material (Fig. 3). Dead removal was not significantly correlated with the number of spores found. Our ability to detect spores was dependant on the number of diseased individuals. We detected one diseased bee in 25.7% of our samples, but 91.4% were positive with 48.2 diseased immatures (Table 3). Negative samples occurred during the 3- and 6-h sampling. Negative samples also were found during the 168-h period when spore levels had dropped. In treatment groups containing 10, 48.2, and 141.0 diseased immatures there was a small, but significant negative correlation ($r = -0.373$, -0.385, and -0.426, respectively, $P < 0.05$; Table 3) between the distance the sample was taken from the diseased comb and the number of spores found in bees. However, when the samples from the diseased comb were removed, no significant correla-

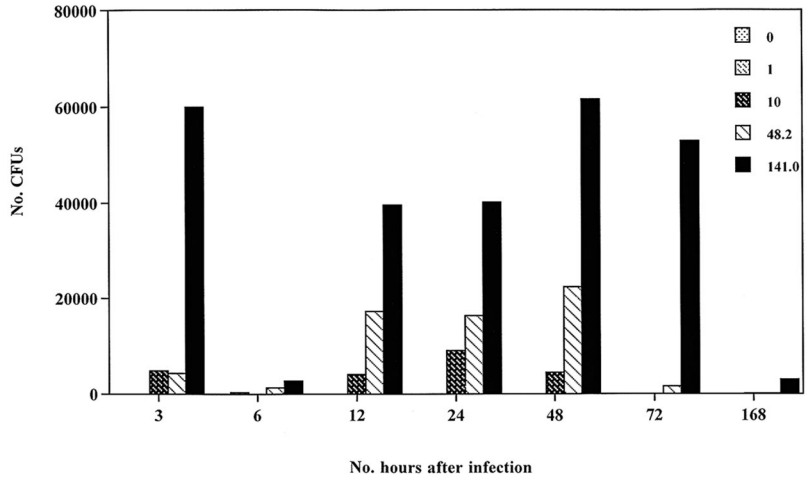

Fig. 2. Number of bacterial CFUs detected in 30 adult bees removed at varying times from the brood nest of healthy honey bee colonies after being artificially infected. Five colonies per treatment group were infected by placing, on average, either 0, 1, 10, 48.2, or 141 diseased immatures in the brood nest. Spores were detected 3 h after infection. By 168 h, spores were either not detected or found at low levels.

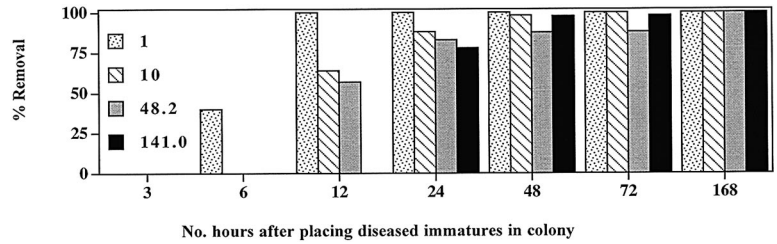

Fig. 3. Diseased brood removal (hygienic behavior) by honey bee colonies infected with 1, 10, 48.2, or 141 diseased immatures ($n = 5$). Time to complete removal was dependent on the number of diseased immatures given.

tion was found. Bees taken from the diseased comb had high CFU levels (mean = 39,182; $n = 21$). Bees from nondiseased combs had significantly lower levels (mean = 6,458 CFUs; $n = 97$; $P < 0.0001$). CFU numbers for the 1, 10, 48.2, and 141 diseased immatures treatments were 48.67, 3,216.65, 11,086.92, and 37,097.37, respectively ($P < 0.05$, $n = 35$ for all means excepting the 48.2 and 141.0 treatments). The colony that received scale tested positive even after intense medication and was destroyed. No spores were detected in control colonies.

On average, 209.45 ± 220.81 CFUs were detected per diseased immature (Table 4). The upper half of positive observations fell within the relatively narrow range of 300–400 spores detected per diseased immature. We define a disease equivalent spore level to be the average number of *P. larvae larvae* CFUs associated with one diseased immature placed in a colony of disease-free bees (adult bee strength and broodnest size described above). A conservative estimate of 1 disease equivalent is the average of the upper 50% of the observations in Table 4, i.e., mean = 399.35 ± 149.72 CFUs. Using this estimator, 22 colonies (3.86%) in the California survey had 1 disease equivalent or more of *P. larvae larvae* CFUs. We detected no disease equivalent levels in colonies from the upper midwestern region (Table 5). The Rocky Mountain region had both the highest spore levels and the highest percentage of colonies with a disease equivalent number of spores (8.73%).

Discussion

California Survey. The cause for regional differences of *P. larvae larvae* spores is unknown. Possibly, frequent colony inspection lowers AFB disease. States in the region with the lowest spore levels (upper Midwest) have active inspection programs. All others excepting Texas and Nebraska have been much less active or were disbanded. States with active inspection do routine health inspections. Owners of colonies with mild cases of the diseased colonies usually are given an opportunity to medicate and restore them to health. Noncompliance often results in burning the colonies. Morse et al. (1965) noted that in New York AFB levels rose and fell with the number of colonies inspected.

Alternatively, resistance to oxytetracycline (OTC) by *P. larvae larvae* caused increased attention to AFB management. We found (unpublished data) the highest level of resistance to OTC in the upper Midwest (area of lowest spore level). Conversely, the lowest OTC resistance was found in the area with the highest level of *P. larvae larvae* spores, i.e., California and the Rocky Mountain states. This suggests that beekeepers in the upper Midwest used prophylaxis more vigorously, thereby reducing spore numbers.

Our conservative estimate of a disease equivalent number of CFUs indicates that 3.86% of colonies had an active infection. Shimanuki (1990) reported that 1.8% of colonies inspected from 44 states were diagnosed with AFB in 1984. In this study, Florida and

Table 3. Correlation between no. of *P. larvae larvae* bacterial CFUs and distance bee sample taken from infected comb in honey bee colonies given varying numbers of diseased immatures of brood

No. diseased immatures (mean ± SD)	No. observations[a]	% samples positive for *P. larvae larvae* spores	Distance sample taken from infective comb[b] (mean ± SD)	No. *P. larvae larvae* CFUs detected per sample (mean ± SD)	Coefficient between spore no. and sample distance
0	30	0		0	
1 ± 0	30	25.7	1.6 ± 1.3	48.7 ± 286.6a[c]	−0.238ns
10.0 ± 0.7	30	57.1	1.7 ± 1.2	3216.6 ± 6,259.7b	−0.375*
48.2 ± 4.8	30	91.4	1.8 ± 1.4	11,086.9 ± 22,744.8c	−0.385*
141.0 ± 41.9	30	82.9	1.6 ± 1.0	37,097.4 ± 61,276.9c	−0.426*
50 scale	7	85.7	1.8 ± 1.0	4,646.9 ± 7,016.9	

To estimate the number of bacterial spores, data should be multiplied by about a factor of 20.

[a] Observations pooled across all sample times of 3, 6, 12, 24, 48, 72, and 168 h.

[b] Distance measured in standard frame spacing, i.e., if sample was taken on frame adjacent to infected comb it was scored as 1.0 frame distant. If sample was taken from comb directly above infected comb it was scored as 1.5 frames distant.

[c] Means followed by the same letter are not significantly different ($P > 0.05$) as determined by LSD.

* Correlation coefficients accompanied with an asterisk were significant ($P < 0.05$).

Table 4. Mean number of *P. larvae larvae* CFUs detected per diseased immature placed in a two-story honey bee colony, spring 2003

No. hours after infecting colonies	Ave no. of *P. larvae larvae*-diseased immatures/colony (n = 5 colonies per treatment group)			
	1 ± 0 (mean ± SD)	10 ± 0.1 (mean ± SD)	48.2 ± 4.8 (mean ± SD)	141.0 ± 41.9 (mean ± SD)
3[a]	0.20 ± 0.30	288.8 ± 645.4	94.43 ± 150.03	465.17 ± 676.53
6	339.2 ± 758.5	0.03 ± 0.04	28.79 ± 44.00	17.00 ± 26.47
12	0.7 ± 1.3	406.57 ± 907.53	355.38 ± 256.53	311.74 ± 472.17
24	0	882.47 ± 787.59	323.97 ± 532.55	325.11 ± 591.02
48	0	408.77 ± 749.70	457.53 ± 938.96	351.80 ± 529.41
72	0.2 ± 0.3	13.42 ± 29.97	295.21 ± 503.63	379.11 ± 204.09
168	0.4 ± 0.5	101.49 ± 226.13	1.41 ± 1.79	15.82 ± 31.80

[a] All colonies were sampled 4 wk before infection and found negative. Disease equivalent equals 399.35 ± 149.72 CFUs. Disease equivalent number of CFUs based on the upper 50% of observed CFUs.

California, which have ≈20% of the total managed colonies, reported a 1.0% infection rate since ≈1970. In our survey, 2.14% of colonies in California had a disease equivalent number of spores. During 1997–2001, Ohio inspected ≈77% of its colonies and found 2.0% colonies infected (Smith 1998, 1999, 2000, 2001, 2002). Florida found 0.9% during the same 5-yr period. Infection rates in other states with large numbers of bees included Minnesota (2.5%), Mississippi (0.5%), North Dakota (0.3%), and South Dakota (1.8%). These are records of visual inspections with an unmeasured error rate.

Spore Recovery from Spiked Samples. About a fourfold increase in CFU detection occurred when bees were macerated. We speculate that the contents of disrupted tissue caused additional spores to germinate. This effect was not as pronounced when bees were crushed with a rolling pin. Further increases in detection would be desirable for survey work. However, there is some optimum sensitivity beyond which gains may not be useful. A technique that detected all spores would likely increase the amount of culturing required for accurate counts without increasing the predictive value of them.

Spore Detection in Artificially Infected Colonies. The cause for the high spore count associated with uncapping is unknown. Gochnauer (1981) found high spore numbers associated with the caps of AFB-infected colonies, so perhaps mouthparts became highly contaminated or caps were eaten. Neither do we do know why there was a drop in spore counts at the 6-h sample (Fig. 2). Possibly, a relatively small group of undertaker-like workers (Breed et al. 2002) began eating the diseased remains at that time. Cleaning time increased as the number of dead increased, suggesting that a relatively small number of bees perform this duty. Spore levels rose after the 6-h sampling, but by 168 h it fell again, indicating that the colony had ridded itself of many spores. Wilson (1971) found after 67 h that most *P. larvae larvae* spores were in the hindgut.

Colonies with AFB scale but receiving prophylaxis are probably not distinguishable from colonies with active infections. Both have large numbers of spores circulating in the colony. (Goodwin and Van Eaton 1999). Either can have serious health risks for the colony. The relatively uniform number of spores detected per diseased immature placed in disease-free colonies allowed the calculation of a disease equivalent number of spores. In natural AFB cases, this number will shift depending on epidemiological factors, e.g., drifting bees, robbing, hygienic behavior, genetic susceptibility, and beekeeper management.

Our error rate decreased with increased numbers of diseased immatures. We detected high levels of disease 80–90% of the time, but only 25.7% of samples were positive with one diseased cell. High spore

Table 5. Number of honey bee colonies with a disease equivalent no. of *P. larvae larvae* bacterial CFUs in the California survey

Region/state	No. colonies with ≥1 disease equivalent of *P. larvae larvae* CFUs	% colonies with ≥1 disease equivalent	No. colonies based on CDFA checkpoint data[a]	Projected no. of colonies with ≥1 disease equivalent
Rocky Mountain[b]	11	8.73	125,816	10,984
California	5	2.14	645,303[c]	13,810
Southwest[d]	2	6.67	27,294	1,821
Northwest[e]	2	3.70	245,172	1,671
Central[f]	2	6.67	7,687	513
Upper Midwest[g]	0	0	268,948	0

Disease equivalent spore number was 399.35 ± 149.72 CFUs.
[a] California colony count estimated by subtracting out of state colonies from 1.4 million total.
[b] Colorado, Montana, Utah, and Wyoming.
[c] California Department of Food and Agriculture 2003 truck entry data.
[d] Arizona and Texas.
[e] Washington, Oregon, and Idaho.
[f] Missouri, Nebraska, Kansas.
[g] North Dakota, South Dakota, and Minnesota.

counts were found in, 3.89% of surveyed commercial colonies. Based on this information, we project that ≈28,799 colonies had 1 disease equivalent number or more of *P. larvae larvae* CFUs. If a 15% error factor is included, the number rises to 33,118 colonies. Many, if not most, of these colonies probably had active cases of AFB. Others not exhibiting AFB, but with high or moderately high spore levels, suggest that prophylaxis must be used to avoid disease outbreaks.

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